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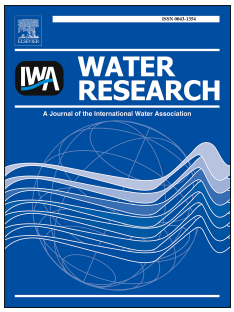

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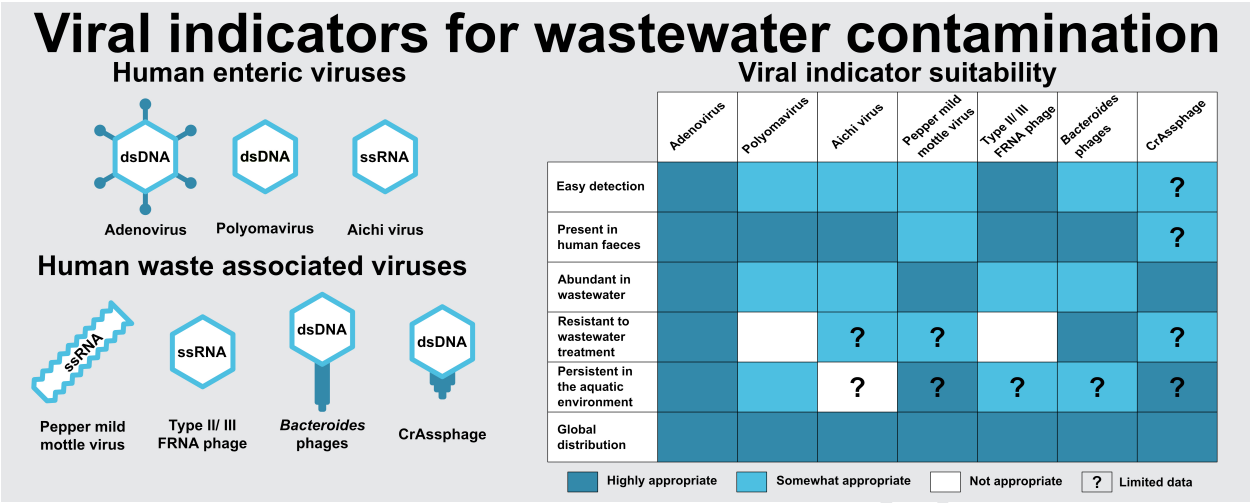

Viral indicators for tracking domestic wastewater contamination in the aquatic environment

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Abstract

Waterborne enteric viruses are an emerging cause of disease outbreaks and represent a major threat to global public health. Enteric viruses may originate from human wastewater and can undergo rapid transport through aquatic environments with minimal decay. Surveillance and source apportionment of enteric viruses in environmental waters is therefore essential for accurate risk management. However, individual monitoring of the >100 enteric viral strains that have been identified as aquatic contaminants is unfeasible. Instead, viral indicators are often used for quantitative assessments of wastewater contamination, viral decay and transport in water. An ideal indicator for tracking wastewater contamination should be (i) easy to detect and quantify, (ii) source-specific, (iii) resistant to wastewater treatment processes, and (iv) persistent in the aquatic environment, with similar behaviour to viral pathogens. Here, we conducted a comprehensive review of 127 peer-reviewed publications, to critically evaluate the effectiveness of several viral indicators of wastewater pollution, including common enteric viruses (mastadenoviruses, polyomaviruses, and Aichi viruses), the pepper mild mottle virus (PMMoV), and gut-associated bacteriophages (Type II/III FRNA phages and phages infecting human *Bacteroides* species, including crAssphage). Our analysis suggests that overall, human mastadenoviruses have the greatest potential to indicate contamination by domestic wastewater due to their easy detection, culturability, and high prevalence in wastewater and in the polluted environment. Aichi virus, crAssphage and PMMoV are also widely detected in wastewater and in the environment, and may be used as molecular markers for human-derived contamination. We conclude that viral indicators are suitable for the long-term monitoring of viral contamination in freshwater and marine environments and that these should be implemented within monitoring programmes to provide a holistic assessment of microbiological water quality and wastewater-based epidemiology, improve current risk management strategies and protect global human health.

Keywords: gastroenteric viruses; environmental sampling; viral indicators; sewage contamination; risk assessment

1. Introduction

1.1 Waterborne enteric viruses

Waterborne diarrheal diseases account for approximately 4 billion cases annually, resulting in 2 million deaths, most of which occur in children under five (WHO, 2010). A significant proportion of these illnesses are caused by enteric viral infections (Ramani and Kang, 2009). Enteric viruses are transmitted via the faecal-oral route and the most important route of transmission is direct contact with infected individuals (Katayama and Vinje, 2017). Nonetheless, most enteric viruses are persistent in environments affected by domestic wastewater discharge and are often associated with waterborne outbreaks (Gibson, 2014; Kauppinen et al., 2018; Sekwadi et al., 2018). Wastewater often receives treatment prior to release into the environment, although traditional wastewater treatment methods can be relatively ineffective at removing enteric viruses (Kitajima et al., 2014; Qiu et al., 2015; Sidhu et al., 2017b). In developing countries, many areas lack adequate sanitary infrastructure and wastewater treatment facilities and hence faecal matter contaminates the environment and drinking water sources (Bain et al., 2014). Furthermore, large volumes of untreated wastewater may also be discharged via combined sewer overflows (CSOs) during heavy rainfall events and via dry water overflows for example during snowmelt, tidal infiltration or system failures and blockages (Ahmed et al., 2020). These events enable the direct entry of enteric pathogens into the environment (Fong et al., 2010), where people in direct or indirect contact with contaminated waters may be at risk of acquiring viral infections (Sinclair et al., 2009). Enteric viruses are readily transported in environmental waters and can adsorb to solid matter present in the water column or accumulate in sediment (Hassard et al., 2016). Subsequently, they may also be taken up by filter feeding aquatic animals such as bivalve shellfish that are harvested for human consumption (Landry et al., 1983; Lowther et al., 2012). Furthermore, wastewater is often used for irrigation in countries experiencing freshwater shortage, and hence, enteric viruses may directly contaminate fruit and salad vegetables and result in foodborne outbreaks (Bosch et al., 2016; Chatziprodromidou et al., 2018; Jasim et al., 2016).

Enteric viruses usually cause gastroenteritis lasting for 2-5 days. In some cases, the infection results in respiratory, neural or epidermal symptoms, or remains asymptomatic (Table 1). The viruses most often

associated with gastroenteritis are members of the *Picornaviridae*, *Caliciviridae*, *Reoviridae* and *Adenoviridae* families (Table 1). For example, noroviruses (family *Caliciviridae*) are responsible for a high proportion of gastroenteritis infections globally, with 685 million cases and approximately 200,000 deaths (CDC, 2016; Katayama and Vinje, 2017), resulting in a total direct cost of US$4.2 billion to the healthcare system and US$60.3 billion in associated societal costs per year (Bartsch et al., 2016). Rotaviruses (family *Reoviridae*) and group F mastadenoviruses (AdVs) (family *Adenoviridae*) are the main causative agents of gastroenteritis amongst infants and young children (Desselberger and Gray, 2009; Jiang, 2006). Noroviruses, hepatitis A virus (family *Picornaviridae*) and AdVs are the most common viral pathogens associated with waterborne and water-associated foodborne outbreaks and infection may result in serious illness, e.g. acute hepatitis (Bellou et al., 2013; Harris et al., 2006; Jiang, 2006; Parshionikar et al., 2003; Sinclair et al., 2009).

Rotaviruses, enteroviruses, sapoviruses, astroviruses, Aichi virus (AiV) and hepatitis E virus are also often shown to be associated with wastewater contamination. For example, in Maharashtra state, India in 2017, a rotavirus B outbreak with a 22.8% attack rate (i.e. new cases/number of people) was sourced from contaminated wells used for drinking water (Joshi et al., 2019). In addition, several viral gastroenteritis outbreaks linked to sewage-contaminated drinking water containing AdV, noro- sapo-, astro-, rota-, and enteroviruses have been reported (Kauppinen et al., 2019; Maunula et al., 2009; Rasanen et al., 2010). The largest viral waterborne outbreak affecting approximately 80,000 people in Kanpur, India was associated with hepatitis E virus (Naik et al., 1992). The surveillance of enteric viral illnesses can be challenging, as many of the enteric viral outbreaks are unreported as the symptoms are often subclinical (Cortez et al., 2017; Koff, 1992; Li et al., 2017; Matson et al., 1993; Sakai et al., 2001; Zaoutis and Klein, 1998).

Over the last decade, both newly discovered viruses and known viruses that had previously not been associated with wastewater have been found in environmental waters (Table 1). Human polyomaviruses (PyVs) and papillomaviruses were first discovered in the 1970s and 1950s, respectively, however, they have only recently been found in the faeces and urine of infected individuals (Knowles, 2006; Rachmadi et al., 2016). Some PyVs, including BKPyV, WUPyV, KIPyV, MCPyV and JCPyV have been detected at high

concentrations (up to 10^8 genome copies (gc)/l) in wastewater, river and seawater and sediment, in swimming pools and in tap water (Di Bonito et al., 2017; Dias et al., 2018; Farkas et al., 2018a; Fratini et al., 2014; Hamza and Hamza, 2018; Rachmadi et al., 2016). As these viruses are commonly asymptomatic in healthy individuals, the route of transmission is not yet clear, however, waterborne infections are likely (Fratini et al., 2014).

Bocaviruses (family *Parvoviridae*), causing respiratory tract infections and gastroenteritis, were first described in 2005 (Allander et al., 2005). They have since been found in untreated and treated wastewater at concentrations of 10^3-10^5 genome copies (gc)/l (Hamza et al., 2017; Iaconelli et al., 2016; Myrmel et al., 2015), however, their prevalence in environmental water has not been explored. The torque teno virus (family *Anelloviridae*), which causes gastroenteritis has also been found in wastewater and in polluted river waters. Similar to bocaviruses, torque teno virus concentrations are considerably lower (up to 10^6 gc/l) than the concentrations of other, more common enteric viruses (10^4-10^9 gc/l) (Hamza et al., 2011; Haramoto et al., 2008). Human picobirnaviruses (family *Picobirnaviridae*) have also been detected in wastewater (concentration range: 10^3 – 10^6 gc/l) and in contaminated rivers with variable prevalence (Adriaenssens et al., 2018; Hamza et al., 2011; Symonds et al., 2009). In addition, recent comparative genomics analysis has suggested that picobirnaviruses are bacteriophages, likely associated with mammalian gut bacteria (Krishnamurthy and Wang, 2018). Genomes or partial genomes of circoviruses (family *Circoviridae*) and cardioviruses (family *Picornaviridae*) along with enveloped viruses (coronaviruses, influenza virus) have also been found in wastewater (Bibby and Peccia, 2013; Blinkova et al., 2009; Ng et al., 2012). Enveloped viruses degrade in water rapidly (Gundy et al., 2009; Lebarbenchon et al., 2011), hence, human infections from waterborne corona- and influenza viruses (e.g. SARS-CoV-2) are unlikely.

1.2 Viral indicators for wastewater contamination

Over 100 types of human enteric viruses are known to be common water pollutants (Melnick, 1984) and with novel and emerging strains, the number is increasing. Due to the diversity of human pathogenic viruses in the environment, surrogates and indicators are often used to investigate the fate and transport of pathogenic strains in the environment. An indicator may be suitable for a broad assessment of

wastewater and drinking water treatment efficiency and for studying pathogen abundance, persistence, adsorption and transport in the aquatic environment. Furthermore, quantitative monitoring of viral indicators can provide useful data for microbial source tracking, transport modelling and risk assessment. Traditionally, faecal indicator bacteria (FIB; including coliform bacteria, *Escherichia coli, Enterococcus* and *Streptococcus* spp.) have been used to determine levels of faecal contamination in water. However, it has been shown that bacteria are significantly less resistant to wastewater treatment and less persistent in the environment than enteric viruses (Fong et al., 2005; Kim et al., 2009; Lin and Ganesh, 2013; Prez et al., 2015; Sidhu et al., 2017a; Staley et al., 2012). Consequently, FIB are poor indicators of viral infection risk and this suggests that current water quality monitoring programmes based solely on FIB are inadequate.

Ideally, a good viral indicator for wastewater-contamination assessment should have similar inactivation and retention to the target pathogens and should be present in wastewater and in wastewater-contaminated environments throughout the year. That would enable continuous monitoring and inform on the level of contamination and the probability of pathogen presence. Furthermore, an indicator with constant levels in wastewater may serve as a proxy for population size when wastewater-based epidemiology is used to estimate the proportion of infected people during a viral outbreak or pandemic, e.g. COVID-19 (Xagoraraki and O'Brien, 2020). Additionally, it should be source-specific to distinguish between animal- and human-derived pollution (Scott et al., 2002). Some enteric viruses associated with wastewater (as listed in Table 1) have potential to be used as indicators, however, not all of those viruses fulfil these requirements. Influenza viruses, coronaviruses, circoviruses and papillomaviruses have been detected at high concentrations in wastewater but not in polluted environments, which may be due to their rapid decay in water. Furthermore, some enteric viruses (e.g. astrovirus, rotavirus, torque teno virus and hepatitis E virus; Table 1) may be zoonotic, hence their presence in the environment may be a result of e.g. agricultural activities instead of human waste. Hepatitis A and E viruses are abundant in less economically developed countries, however, they are only responsible for sporadic outbreaks in more developed regions (Bosch et al., 2016). Further, enteroviruses, noroviruses and sapoviruses show clear seasonality with peaks either in the summer (enteroviruses) or during the winter (noroviruses and sapoviruses) in temperate climates. Hence, these viruses are not found in wastewater and in the contaminated environment at all

155 times of the year (Farkas et al., 2018a; Nino Khetsuriani et al., 2006; Pons-salort et al., 2018; Prevost et al.,
156 2015). Human AdVs, PyVs and AiVs are frequently found in wastewater and in polluted environments
157 without any distinct seasonality, hence their utility as effective faecal indicators have been suggested
158 (Kitajima and Gerba, 2015; Rachmadi et al., 2016; Rames et al., 2016).

159 Bacteriophages infecting bacteria associated with the human gut are also common in wastewater. Somatic
160 coliphages (phages infecting *E. coli*) and F-specific RNA bacteriophages (FRNAP; phages infecting bacteria
161 through the F-pili) are commonly used to assess wastewater contamination. However, as not all strains
162 exclusively associate with human bacteria, they should be used with caution. Bacteriophages infecting
163 *Bacteroides* spp. also have the potential to indicate wastewater contamination. Amongst these phages are
164 a newly discovered group of viruses called crass-like phages. The type genome, crAssphage *sensu stricto*
165 (metagenome-assembled genome), belongs to the normal gut virome, having co-evolved with humans
166 (Dutilh et al., 2014; Edwards et al., 2019). Since the discovery of the first crAssphage genome, more crass-
167 like sequences have been found and one phage has been isolated. However, their genomic diversity is large
168 and the crAssphage *sensu stricto* and the isolated crass-like phage do not belong to the same genus
169 (Shkoporov et al., 2018). As the taxonomy of crass-like phages remains to be established, we refer to
170 crAssphage as a group of viruses with nucleotide similarity to the crAssphage *sensu stricto* described by
171 Dutilh et al. (2014) and quantified by Stachler et al. (2017).

172 Interestingly, a plant virus, the pepper mild mottle virus (PMMoV; family *Virgaviridae*), has also been
173 shown to be associated with human wastewater and found in polluted surface and groundwater and in
174 drinking water (Symonds et al., 2018). The primary source of PMMoV in human excreta is through
175 consumption of peppers (*Capsicum* spp.) and food products containing peppers that are contaminated with
176 the virus (Zhang et al., 2005). PMMoV is suggested to be a useful indicator for wastewater contamination
177 (Kitajima et al., 2018b; Symonds et al., 2018), however, its shape and size (17 x 300 nm rod-shaped capsid)
178 differs from other pathogenic viruses with icosahedral capsids and hence its fate and behaviour in the
179 environment may be different.

In this review, we evaluated the practicality of a set of human-waste associated viruses as indicators for wastewater contamination of the aquatic environment (Table 2). We have extracted data from 127 individual studies to assess the usefulness of viral indicators by addressing specific aspects. The data collected on viral concentrations in wastewater and environmental receiving waters are presented in Tables S1-6, while the corresponding wastewater treatment log removal rates for each virus are presented in Table S7. Together, we used this information to assess the ranges of virus abundance and distribution in global aquatic systems. We included human wastewater-associated viruses, which are often present in wastewater at high concentration without seasonality. We considered enteric viruses, (human AdVs, PyVs and AiVs), PMMoV and human gut bacteria-associated bacteriophages, including FRNAP infecting *E. coli* (specifically genogroups II and III), and bacteriophages of human gut commensal *Bacteroides* spp. (including crAssphage). For evaluation, we used the following criteria:

1. Ease of detection and quantification
2. Human waste association
3. Presence in wastewater at high concentrations
4. Resistance to wastewater treatment
5. Persistence in the aquatic environment
6. Global distribution and temporal stability

2. Data collection

We collected viral concentration data published in peer-reviewed journal articles since 2005 (Tables S1-7; Figure 1). Articles were identified via Google Scholar in September 2018 – October 2019 using the following keywords: 'wastewater adenovirus', 'wastewater polyomavirus', 'wastewater Aichi virus', 'crAssphage', 'wastewater pepper mild mottle virus', 'wastewater AND ("F-specific RNA" OR F+ OR "FRNA" OR "male specific") AND *phage AND genogroup' and 'wastewater *Bacteroides* bacteriophage''. The Google Scholar search included these terms or part of them in the whole text, hence enabling the identification of studies on the aquatic environment where wastewater contamination was assessed using the target viruses. The studies were screened based on the title and abstract and initially 243 papers were selected.

The assessment of enteric viruses, PMMoV and crAssphage concentrations usually involved the concentration of large volumes of water (1-10 l), and the efficiency of those procedures may therefore affect the outcomes. Hence, studies where viral concentrations were not determined or sample process recovery efficiency and/or quantitative PCR (qPCR) performance was not addressed were excluded from the study. Studies where sample process recovery was <10% were also excluded. After the quality screen, 127 peer-reviewed research papers were included in the review (Table 2). Viral concentration data were classified by water type (untreated and treated wastewater, surface freshwater, groundwater and seawater) and the detection rates (i.e. positive samples / all samples x 100%), mean/median concentrations and/or minimum-maximum concentrations were extracted (Figure 2, Table S1-S6). In most studies only the mean/median and/or minimum-maximum concentrations were reported, hence further meta-analysis was not performed. Virus removal rates reported during wastewater treatment processes were also retrieved (Figure 3; Table S7). Additionally, the primers and probes used for the qPCR detection and quantification of viruses have also been summarised (Table S8).

3. Evaluation of viral indicators

3.1 Criterion 1: Ease of detection and quantification

For the accurate detection of low viral titres, environmental samples are often concentrated prior to virus detection. Ultracentrifugation, ultrafiltration, adsorption/elution and flocculation are often used for the concentration of water samples, and their effectiveness and limitations have been reviewed previously (Barardi et al., 2012; Bofill-Mas and Rusiñol, 2020; Cashdollar and Wymer, 2013; Haramoto et al., 2018; Ikner et al., 2012). The efficiency of viral recovery depends on the type of concentration method used, the sample type and the virus type. Hence, viruses that can be easily and reproducibly recovered using simple concentration methods should be used as indicator viruses.

Many approaches are available for the detection and quantification of viruses in environmental samples, including PCR and isothermal amplification of target genes, microfluidics, metagenomics, biosensors, microarrays and culturing-based techniques, as reviewed recently (Bonadonna et al., 2019; Farkas et al.,

2020; Hamza and Bibby, 2019). Many of the emerging approaches show great potential to detect low concentrations of viruses in difficult matrices (Dhar and Lee, 2018; Farkas et al., 2020; Gyawali et al., 2019b), however, to date they have not been implemented in the monitoring of viral contamination in the aquatic environment.

In most studies, enteric viruses and proposed indicators were detected and quantified using real-time quantitative PCR (qPCR)-based approaches (Girones et al., 2010; Haramoto et al., 2018), which are rapid, easy, and cheap methods enabling strain-level detection. For instance, targeting different regions of the hexon capsid protein gene, all human AdVs or only enteric AdVs (AdV genogroup F) can be quantified (Table S1 and S8). qPCR can be easily multiplexed, enabling the simultaneous detection of 2-5 viral targets (Ahmed et al., 2019a; Farkas et al., 2017b; Jiang et al., 2014; Lee et al., 2016; Montazeri et al., 2015). Hence, it is widely used for the analysis of the level and spread of viral contamination in the aquatic environment (Staggemeier et al., 2017). More recently, digital PCR approaches, enabling absolute quantification without relying on standards, have also been used for the estimation of viral counts in wastewater and in environmental waters (Ishii et al., 2014; Jumat et al., 2017; Kishida et al., 2014; Sedji et al., 2018). These methods are also efficient, sensitive and often provide more accurate results that qPCR (Ishii et al., 2014; Kishida et al., 2014).

The primers and probes repeatedly used in environmental studies for the detection and quantification of the potential indicator viruses with qPCR, reverse transcription (RT) qPCR, and dPCR, are listed in Table S8. In general, hydrolysis probe-based assays were predominantly used for viral detection. The specificity and sensitivity of the primer and probe sets had been assessed (empirically or *in silico*) using a set of target and non-target sequences and dilution series and shown to be adequate for the quantification of the target sequences (Barrios et al., 2018; Chehadeh and Nampoory, 2013; Dumonceaux et al., 2008; Goh et al., 2009; Gröndahl et al., 1999; Heim et al., 2003; Hernroth et al., 2002; Jothikumar et al., 2005; Kitajima et al., 2013; Ko et al., 2005; McQuaig et al., 2009; Ogorzaly and Gantzer, 2006; Pal et al., 2006; Pang et al., 2012; Prevost et al., 2015; Rusiñol et al., 2015; Stachler et al., 2017; van Maarseveen et al., 2010; Wolf et al., 2010, 2008; Xagoraraki et al., 2007). The high detection rates (Figure 2; Table 2) also suggest that the primer and probe

sets were suitable for the sensitive detection of the target viruses. Nonetheless, the specificity of the sets should be revised frequently in order to assure that novel strains are detected.

PCR-based approaches however have some disadvantages. qPCR and especially RT-qPCR are often inhibited by organic substances, e.g. polyphenolic compounds, found in environmental samples (Ahmed et al., 2015; Farkas et al., 2017a; Girones et al., 2010; Matheson et al., 2014). Therefore, the use of DNA viruses as indicators (e.g. AdV, PyV, crAssphage) for wastewater-derived viral contamination may be more feasible than the use of RNA viruses (AiV, PMMoV, FRNAP) due to the more robust molecular detection of DNA targets (Farkas et al., 2017a; Hata et al., 2011). A major disadvantage of all PCR-based viral detection approaches is that they do not give any indication on the infectivity of the target, and hence often overestimate viral concentration and human health risks (Knight et al., 2013). Detecting segments of indicator genes is helpful for the evaluation of the magnitude of faecal contamination and source tracking, nonetheless, an ideal indicator should be capable of being cultured *in vitro* to enable the direct assessment of viral infectivity and decay in wastewater and in the aquatic environment.

In the studies subject to this review, enteric viruses, crAssphage and PMMoV were predominantly detected and quantified using qPCR-based assays. However, in a few studies plaque assay or integrated cell culture-qPCR (ICC-qPCR) were used for AdV detection (Table S1). The combination of cell culture and qPCR detection of viral replication enabled the detection of infectious viruses, which grew slowly and/or failed to produce cytopathic effects. Using this approach, the time required for infectivity analysis has been reduced from one week to two days, enabling rapid detection. Overall, qPCR/dPCR gave 1-5 $\log_{10}$ higher AdV concentrations than plaque assay and ICC-qPCR due to the presence of damaged virus particles and free viral DNA derived from degraded viruses in the environmental samples (Fongaro et al., 2015, 2013; Hamza et al., 2011; Hewitt et al., 2011; Rigotto et al., 2010; Rodríguez et al., 2013; Sassoubre et al., 2012; Sedji et al., 2018). The higher concentrations detected using ICC-qPCR compared to the traditional culturing assays suggest that using qPCR-based quantification of cultured viruses is more sensitive and hence more reliable in environmental settings (Fongaro et al., 2013; Sedji et al., 2018). PyVs and AiV are also culturable, however, the propagation process is time consuming (2-4 and 4-6 weeks, respectively) and often

inconclusive (Reuter et al., 2011; Seehafer et al., 1978), hence, these approaches have not been adapted to environmental studies. ICC-qPCR-based approaches may be suitable for assessing the infectivity of these viruses in the environment, however, to date no ICC-qPCR assays have been developed for these targets. The disadvantage of any culturing-based assay for enteric virus detection is the need for specific equipment (e.g. CO_2 incubator), environment (BSL2 or BSL3) and staff for the maintenance of specific cell lines, which may not be available in routine monitoring laboratories.

FRNAP are easy to culture and readily form plaques on a lawn of the host bacterium, which is usually the WG49 strain of *Salmonella typhimurium* or *Escherichia coli* HS(pFamp)R (USEPA, 2001). Higher volumes (up to 100 ml) of samples or concentrates are typically used for culture-based assays than for qPCR (a few microliters of nucleic acid extract) and so culturing can be more sensitive than direct qPCR. However, this method will produce plaques of a range of different strains that cannot be differentiated based on morphology. Therefore, to identify and quantify specific FRNAP genogroups, it is necessary to use genogroup-specific molecular detection methods. Such methods include RT-PCR analysis of plaques (Haramoto et al., 2015, 2012) and 1-day ICC-RTqPCR (Hartard et al., 2017), most-probable number assays (Hata et al., 2016) or *in-situ* plaque membrane hybridisation techniques (Flannery et al., 2013).

Many *Bacteroides*-associated phages are also culturable using appropriate hosts, including *Bacteroides* strains GB-124, RYC2056, GA17 and ARABA-84, with double-layer agar method to quantify the number of plaque forming units (pfu). However, the assay is more challenging than the plaque assay for FRNAP as culturing *Bacteroides spp.* require anaerobic conditions, which may not be available in most laboratories. The recent isolation and *in vitro* maintenance of phage ΦCrAss001 infecting *Bacteroides* sp. indicates that plaque assays for this type of phage may be used in future environmental studies (Shkoporov et al., 2018). It is important to mention, though, that the crAssphage qPCR assay (Table S7) does not detect ΦCrAss001, as this phage is reported to belong to a different genus.

In order to estimate viral decay where no *in vitro* infectivity assay is routinely available (e.g. hepatitis E virus, noro- and sapovirus), capsid integrity assays can be performed based on the assumption that an

intact virus particle is infectious. Capsid integrity can be assessed by the elimination of free viral nucleic acids using enzymatic treatment, such as DNase, RNase (Fongaro et al., 2013) and intercalating dye pre-treatment (Prevost et al., 2016) or by capturing only the intact virus particles using immunomagnetic separation (IMS) (Haramoto et al., 2010). PCR-based enumeration following integrity assays show lower viral concentrations than direct qPCR, as the free viral nucleic acids are eliminated. However, as the intact virus particles may be damaged and hence non-infectious, these approaches may still overestimate viral counts (Fongaro et al., 2013; Walker et al., 2019). Nonetheless, integrity assays are valuable tools for estimating the number of viral particles in environmental samples and their use may improve viral risk assessment.

3.2 Criterion 2: Human waste association

Viruses, such as AdV, PyV and AiV strains (Table S1-S3), which specifically infect humans, are logical choices for indicators for human faecal contamination. Using these viruses and their corresponding animal associated strains, the source of contamination (e.g. human vs wildlife, livestock, etc.) can be assessed. For example, Staggemeier et al. (2015) used SYBR Green qPCR for the detection and quantification of AdVs in water and sediment samples by distinguishing human, bovine, porcine, canine and avian AdV genome sequences based on their melting temperature. Human and porcine AdVs, bovine PyV and porcine circovirus have also been used to assess the level of agriculture-related and human sewage-associated contamination in recreational, groundwater and drinking water (Fongaro et al., 2015; Garcia et al., 2012; Rusiñol et al., 2014). In the studies evaluated in this review, the AdV qPCR assays targeted all human AdV groups (A-G), the most common groups (A-F) or specific groups (C and F; Table S1). All of these groups are human-specific, demonstrating that waterborne infections of AdV F (predominantly type 41) are the most prevalent in wastewater and in the aquatic environment (Bofill-Mas et al., 2010; Chigor and Okoh, 2012; Fong et al., 2010; Fumian et al., 2013; Haramoto et al., 2007; Hewitt et al., 2011; Iaconelli et al., 2017; Ibrahim et al., 2018; Lun et al., 2019; Myrmel et al., 2015; Ogorzaly et al., 2015; Shih et al., 2017). The most common PyVs associated with wastewater are the JC and BK strains (Table S2), however, MC PyV is also found in wastewater and in wastewater-contaminated water (Di Bonito et al., 2014; Rusiñol et al., 2015).

All known human AiV (group A and B) have been found in human wastewater (Table S3). These viruses are highly human-specific and have not been found to associate with animal diseases. The JC and BK strain have not been found in animal waste (McQuaig et al., 2009), whereas to date, animal waste samples have not been tested for human AiV.

PMMoV has been found at high concentrations in domestic wastewater (raw and treated) and in wastewater-polluted environments and shown to correlate well with other human markers (*Bacteriodes* HF183, PyV) (Kitajima et al., 2018b; Symonds et al., 2018, 2016) implying it associates with human waste. Nonetheless, the primary source of the virus are bell and chilli peppers, with the suggestion that it should not be used as a faecal indicator nearby to commercial pepper plant production areas. qPCR assays targeting PMMoV show high sensitivity, however, the viruses were also detected in avian, bovine and dog faeces at low concentrations (Gyawali et al., 2019a; Hamza et al., 2011; Rosario et al., 2009) suggesting that animals may also access pepper as a food source. Furthermore, it has been suggested that PMMoV is more abundant in faeces and in wastewater where more pepper products are consumed (Symonds et al., 2018), therefore the prevalence of PMMoV should be further investigated.

Coliphages are commonly used as indicators for faecal viral contamination in water (McMinn et al., 2017). FRNAP genogroups II and III (FRNAP-II and FRNAP-III) have been shown to be associated with human sources, while genogroups I and IV (FRNAP-I and FRNAP-IV) are generally associated with non-human sources (Lee et al., 2011; Stewart-Pullaro et al., 2006). For this reason, several studies have used methods (described in Section 3.1) to distinguish between FRNAP genogroups to determine faecal sources. However, while there does appear to be a general association between genogroups and faecal sources, the bacterial host (*E. coli* expressing F-pili) is not source-specific and there is often overlap between source types for each genogroup (Cole et al., 2003; Harwood et al., 2013).

Bacteriophages infecting *Bacteroides*, common human gut bacteria, have also shown potential as indicators for faecal contamination in the environment. The most commonly used strains, which are phages that infect *Bacteroides* BG-124 (BacBG124P), RYC-2056 (BacRYC2056P), GA-17 (BacGA17P) and ARABA-84 (BacARABA84P), were shown to be human specific. However, one study detected BacRYC2056P in

wastewater samples derived from abattoirs, suggesting animal association (Wicki et al., 2015). qPCR based assays targeting crAssphage have shown good human specificity. While some cross-reactivity has been shown with dog, gull, poultry, pig and cattle faeces (Ahmed et al., 2018a; García-Aljaro et al., 2017; Stachler et al., 2017), the levels of crAssphage found in these non-human sources were several orders of magnitude lower than that of human sources. The highest crAssphage concentrations in animal sources were found by García-Aljaro et al. (2017) which may be attributed to pooled samples and/or the use of a different qPCR assay. García-Aljaro et al. (2017) also found that by normalising crAssphage levels against a general faecal indicator (*E. coli*), it was still possible to distinguish between human and non-human sources. Nevertheless, the cross-reactions of the qPCR with animal excreta should be further investigated to assess human specificity.

3.3 Criterion 3: Presence in wastewater at high concentrations

AdVs, PyV, AiV, human gut-associated bacteriophages and PMMoV are all frequently found in raw sewage and untreated wastewater at high concentrations (Figure 2; Table S1-6). The highest concentrations among the potential indicators were noted for crAssphage with concentrations of 10^{10} – 10^{12} gc/l in raw sewage detected in samples taken in Japan. CrAssphage concentrations were lower in wastewater samples taken in the US (Florida; 10^{9} – 10^{10} gc/l) (Ahmed et al., 2018a) and in the UK (Wales; 10^{5} – 10^{8} gc/l) (Farkas et al., 2019). CrAssphage is not currently well characterised and while the current primer and probe set do not align to any recently discovered relatives of crAssphage, it is possible that the qPCR-based detection assay is not specific to a single strain.

AdVs were detected at 10^{11} gc/l concentration in wastewater influent, in Pisa, Italy in 2009-2010 (Carducci and Verani, 2013). In other studies using the same primer and probe set (Hernroth et al., 2002), the concentration of AdV was lower, between 10^{3} and 10^{9} gc/l with the highest concentrations measured in other wastewater treatment plants in Italy (La Rosa et al., 2010), followed by peak concentrations of 10^{8} gc/l in Rome, Italy (Muscillo et al., 2008), Barcelona, Spain (Bofill-Mas et al., 2006), and in Minas Gerais and Rio de Janeiro, Brazil (Assis et al., 2017). Similar peak concentrations (10^{8} gc/l) were observed when AdV groups A-G were targeted in Germany (Hamza et al., 2009a) and in Queensland, Australia (Sidhu et al.,

2017b). The highest concentrations of AdVF (10^8-10^{10} gc/l) were observed in the US (Michigan) (Simmons et al., 2011) and in Giza, Egypt (Elmahdy et al., 2019) further verifying that this group of AdV is highly prevalent.

PMMoV are also present in wastewater at high concentrations (up to 10^{10} gc/l). The highest PMMoV concentrations were reported in Florida and other states in the US (Rosario et al., 2009) followed by Germany (10^7 – 10^8 gc/l) (Hamza et al., 2011), New Zealand (10^7 gc/l) (Gyawali et al., 2019a), Vietnam and the US (Arizona; 10^6 – 10^7 gc/l) (Kitajima et al., 2014; Kuroda et al., 2015; Schmitz et al., 2016). Slightly lower FRNAP concentrations were noted in wastewater with the FRNAP-II appears to be more prevalent (10^7 – 10^9 gc/l) than FRNAP-III (10^4 – 10^7 gc/l) (Figure 2). However, more studies are needed to further investigate FRNAP-II/III concentrations in wastewater.

Among PyVs, JCV had the highest concentrations (10^7-10^8 gc/l) in wastewater collected in Brazil and Chile (Fumian et al., 2010; Levican et al., 2019), however, it was less prevalent (10^3-10^6 gc/l) in the US (Arizona), Spain and in the UK (Wales) (Bofill-Mas et al., 2006; Farkas et al., 2018a; Kitajima et al., 2014; Rusiñol et al., 2015; Schmitz et al., 2016). BKV and MCV are probably less abundant in wastewater than JCV with concentration ranges of 10^3-10^7 gc/l and 10^4-10^5 gc/l, however, limited surveillance has been done on these viruses in wastewater. AiV has only been sought in untreated wastewater in the USA (Arizona), Vietnam and Nepal (Haramoto and Kitajima, 2017; Kitajima et al., 2014; Kuroda et al., 2015; Schmitz et al., 2016) with concentrations between 10^4 and 10^6 gc/l, and shown to be less prevalent than the other indicators. The detection rate and concentration of AdV, PyV, AiV, FRNAP-II, crAssphage and PMMoV usually exceeded the concentration of noroviruses, sapovirus, enterovirus, astrovirus, rotavirus and hepatitis E virus (Farkas et al., 2019, 2018a, 2018b; Flannery et al., 2013; Fumian et al., 2013; Grøndahl-Rosado et al., 2014a; Hata et al., 2014; Kitajima et al., 2014, 2013; Masclaux et al., 2013; Prevost et al., 2015; Qiu et al., 2015; Simmons et al., 2011). However, in some cases norovirus and rotavirus showed higher concentrations than AdV and PyV (Kaas et al., 2018; Prado et al., 2019).

The peak concentrations of cultured phages infecting *Bacteroides* in untreated wastewater was 10^6 pfu/l, however, this number cannot be directly compared with the concentration of other indicators due to the

412 different methods used for detection. The viral infectivity rate (i.e. gc: infective units) of virus may be as
413 high as 1000:1 as determined for AdV (Hewitt et al., 2011), however, the actual infectivity rates for
414 *Bacteroides*-associated phages is yet to be determined.

415 All potential indicator viruses showed high (>90%) detection rates in untreated wastewater, except
416 BacRYC2056P, which was found only in 82% and 38% of the analysed samples. AdVs and PyVs were
417 frequently detected in wastewater from large and small wastewater treatment plants in the Americas,
418 Europe, Asia and Australia (Figure 1; Table 2; Table S1, S2). Prevalence and concentration information for
419 AiV was only reported for untreated wastewater samples from large wastewater treatment plants in the
420 USA (Arizona) and Vietnam (Kitajima et al., 2018a, 2014, 2013; Kuroda et al., 2015; Schmitz et al., 2016).
421 The PMMoV titre was assessed in wastewater samples derived from the USA, Germany, New Zealand and
422 Vietnam (Hamza et al., 2011; Kitajima et al., 2014; Kuroda et al., 2015; Rosario et al., 2009; Schmitz et al.,
423 2016; Symonds et al., 2016). FRNAP-II prevalence in wastewater was only assessed in Japan and Ireland
424 (Flannery et al., 2013; Haramoto et al., 2015, 2012; Lee et al., 2018), while FRNAP-III prevalence in
425 wastewater was only assessed in Japan (Haramoto et al., 2015, 2012; Lee et al., 2018; Setiyawan et al.,
426 2014, 2013). In wastewater, the concentrations of phages associated with *Bacteroides* BG-124 were
427 assessed in the US, Brazil, the UK (England) and Switzerland (E. Dias et al., 2018; Mcminn et al., 2014; Prado
428 et al., 2018; Purnell et al., 2015; Stefanakis et al., 2019; Wicki et al., 2015), BacRYC2056P were investigated
429 in Colombia, the UK, Spain, France, Cyprus, Sweden, Switzerland and Thailand (Costán-Longares et al.,
430 2008; Gomila et al., 2008; Payan et al., 2005; Venegas et al., 2015; Wangkahad et al., 2017; Wicki et al.,
431 2015, 2011), BacGA17P were found in Colombia and several European countries (Casanovas-Massana et al.,
432 2015; Costán-Longares et al., 2008; Gomila et al., 2008; Mayer et al., 2016; Payan et al., 2005; Venegas et
433 al., 2015; Wicki et al., 2015) and BacARABA84P was identified in Switzerland (Wicki et al., 2015, 2011).
434 CrAssphage concentrations were only determined in wastewater in the UK (Wales), Australia and the USA
435 (Florida) (Ahmed et al., 2019a, 2018b; Farkas et al., 2018a). Due to the limited number of studies (Table 2),
436 further testing is necessary to evaluate the prevalence and distribution of AiV, FRNAP-II, FRNAP-III,
437 culturable phages infecting *Bacteroides*, crAssphage and PMMoV in untreated wastewater to assess their
438 usefulness as indicators of wastewater pollution. Current data suggest that all assessed viral indicators, are

present in untreated wastewater at high concentration and hence they are potentially good indicators for wastewater contamination.

3.4 Criterion 4: Resistance to wastewater treatment

Enteric viruses have been shown to be extremely resistant to traditional wastewater treatment procedures (Figure 3; Table S7). As the removal efficiency varies amongst sites and the type of treatment process, comparative studies have been performed to study the resistance of enteric viruses and potential indicators during wastewater treatment.

In this study, 24 studies comparing the virus removal efficiency of different wastewater treatment processes were evaluated (Figure 3). Fifteen of these studies exclusively used qPCR and RT-qPCR for the quantitative analysis of viral concentrations. As discussed in Section 3.1, these molecular techniques give no indication on the infectivity state of the viruses and hence may overestimate infective viral titres in untreated and treated wastewater and other environmental samples. This was demonstrated by Flannery et al. (2013a) whose data showed that while infectious FRNAP-II in UV-treated effluent was approximately 2.3 $\log_{10}$ less than influent, only a 0.54 $\log_{10}$ reduction was found when using RT-qPCR alone. Most of this reduction in FRNAP-II infectivity occurred during the secondary treatment stage (1.69 $\log_{10}$ reduction), but the type of secondary treatment used in that study was not specified. In a study by Lee et al. (2018), an activated sludge process (a form of secondary treatment) resulted in 2.1 and 3.1 $\log_{10}$ reductions of infectious FRNAP-II and FRNAP-III, respectively. RT-qPCR analysis of the same samples showed $\log_{10}$ reductions of 1.6 and 2.5 for FRNAP-II and FRNAP-III, respectively. The differences between infectious virus and genome removal were not significant. These studies therefore reached conflicting conclusions with the former showing that infectivity studies are vital and the latter showing that they are unnecessary. It is possible that the activated sludge process used by Lee et al. (2018) resulted in physical removal of viruses, while the process used by Flannery et al. (2013a) inactivated the viruses without physically removing them from the treated water. This highlights the importance of including the specific mechanisms used within a sewage treatment process when reporting such data, as it is not clear whether the secondary treatment processes in the two studies shared any mechanistic similarities.

Low removal rates have been reported for BacGB124P and BacGA17P during wastewater treatment. In most studies, the removal of these phages was ≤ 2 log_{10}, regardless of the treatment method used (Dias et al., 2018; Mayer et al., 2016; Prado et al., 2018; Stefanakis et al., 2019), except for one study showing >5.6 log_{10} removal of BacGB124P when disk filtration and chlorination was used as tertiary treatment (Prado et al., 2018).

Data obtained from a wide range of qPCR-based viral quantification studies have shown limited removal of AdV, PyV, AiV, crAssphage and PMMoV during wastewater treatment. Activated sludge treatment and biofiltration, without further treatment resulted in 0.6-1.9 and 0.3-3.0 log_{10} removal of AdV and PyV, respectively (Figure 3; Table S7). Tertiary treatment processes resulted in an additional 1-3.5 log_{10} removal of AdV and PyV with membrane bioreactors coupled with additional chlorination, filtration and UV treatment being the most efficient method for viral removal (Qiu et al., 2018; Rusiñol et al., 2015; Simmons et al., 2011). Furthermore, AdVs have been shown to be more resistant to UV treatment than poliovirus, rotavirus, caliciviruses and hepatitis A virus (Hijnen et al., 2006). However, laboratory-scale studies suggest that AdVs are more susceptible to chlorine treatment than enteroviruses and caliciviruses (Cromeans et al., 2010; Kahler et al., 2010; Thurston-Enriquez et al., 2005, 2003). Interestingly, significant differences were found for the removal of PyV strains. MCV was found to be the most resistant to treatment followed by JCV and BKV (Rusiñol et al., 2015).

Fewer studies evaluated the removal of AiV, crAssphage and PMMoV, than the removal of AdVs, PyVs and phages. Overall, AiV showed 1-3 log_{10} reduction during secondary and 1-2 log reduction during tertiary wastewater treatment (Kitajima et al., 2018a, 2014, 2013; Schmitz et al., 2016). The removal of crAssphage was also in the range of 1.0-1.2 log_{10} during secondary wastewater treatment (Farkas et al., 2019), however, crAssphage removal has not been assessed during tertiary treatment yet. The current data suggests that PMMoV is stable during secondary treatment and chlorination, which results in <2 log reduction (Symonds et al., 2018). Larger PMMoV removal (>4 log) was only observed using electrocoagulation and Bardenpho (aerobic/anaerobic multi-reactor) technologies (Schmitz et al., 2016; Symonds et al., 2015). Further research is needed to evaluate the reduction of PMMoV during UV

treatment and other wastewater treatment procedures to evaluate its usefulness as an indicator. The major advantage of crAssphage and PMMoV is that their concentrations are usually high in wastewater, and hence the efficiency of their removal can be easily monitored. Nonetheless, their infectivity and decay have not been investigated due to the current lack of i*n vitro* culturing-based methods.

In studies where the removal of indicator viruses was compared with the removal of common pathogenic enteric viruses, indicator viruses showed similar or less removal than the pathogens (Carducci and Verani, 2013; Farkas et al., 2018a; Kitajima et al., 2014; Prado et al., 2019; Rusiñol et al., 2015; Schmitz et al., 2016). Furthermore, a meta-analysis on the efficiency of secondary wastewater processes showed that activated sludge treatment resulted in 0.20 – 2.18 $\log_{10}$ reduction of rotavirus, and norovirus GI and GII, whereas biofiltration resulted in higher removal (1.52 – 4.30 $\log_{10}$) of norovirus GII and enteroviruses (Sano et al., 2016). These removal rates are higher than the removal rates determined for the indicators reviewed here suggesting that the indicators can represent the removal of the most resistant viruses. However, three studies showed higher removal rates of BKV and JCV than norovirus, sapovirus, enterovirus and rotavirus (Farkas et al., 2018a; Fumian et al., 2013; Schmitz et al., 2016) suggesting that PyVs are less resistant than the pathogenic viruses and hence should be used with caution as an indicator. Current data shows that AdV, AiV, FRNAPII/III, crAssphage and PMMoV may be suitable for the assessment of wastewater treatment processes.

As different viruses have varying reactions to wastewater treatment processes, the use of multiple indicators is recommended. For indicators other than bacteriophages reviewed here, the exclusive use of molecular detection and quantitation is a major limitation in understanding enteric virus removal. Hence, combinations of infectivity studies and molecular assays should be performed for viruses that can be cultured *in vitro* in order to assess viral survival during wastewater treatment.

3.5 Criterion 5: Persistence in the aquatic environment

Many of the studies that have been conducted to estimate viral persistence in natural waters have relied solely on qPCR-based quantification. While the reliance on qPCR data alone may lead to overestimations of infectious viral persistence, its use is nonetheless important especially when considering unculturable

enteric viruses. When measuring persistence of viral indicators in the aquatic environment, researchers should therefore be clear whether they are studying the persistence of a viral signal (for example nucleic acids detected by qPCR) or the infectivity of viruses (for example by culture).

3.5.1 Indicators in surface freshwater

Most research has focused on the occurrence and survival of indicator viruses in surface water. When quantified in surface freshwater bodies (lakes, rivers, streams, etc.) by qPCR, these viral indicators (e.g. AiV, AdV, JCV, PMMoV) are typically detected at up to 4 $\log_{10}$ higher concentrations than common enteric pathogenic viruses, e.g. norovirus, enterovirus and rotavirus (Hata et al., 2014; Jurzik et al., 2010; Rusiñol et al., 2015; Sassi et al., 2018). All indicator concentrations in river water correlated with the distance of sampling point from the source of contamination (wastewater treatment plant), with significantly higher concentrations occurring near the wastewater treatment plant than further downstream or upstream (Ebdon et al., 2007; Farkas et al., 2018a; Prevost et al., 2015; Rusiñol et al., 2015; Sassi et al., 2018; Sibanda and Okoh, 2012; Tandukar et al., 2018; Venegas et al., 2015; Wangkahad et al., 2017). Comparative studies showed that PMMoV occurred at higher concentration than AdV, AiV and PyV in surface water bodies in the USA (Arizona: 10^3-10^6 gc/l and Colorado: 10^4-10^5 gc/l), Germany (10^4-10^5 gc/l) and Vietnam (10^4-10^6 gc/l)(Betancourt et al., 2014; Hamza et al., 2011; Kuroda et al., 2015; Sassi et al., 2018). The concentration of AdV and AiV (up to 10^4 gc/l) were similar in river water collected in the USA (Colorado) and Japan (Betancourt et al., 2014; Hata et al., 2014; Sassi et al., 2018), whereas AdV was more prevalent than PyV in river water samples collected in Spain (76% vs 48% detection rates), the UK (Wales: 88% vs 65%), Japan (61% vs 11%) and Germany (79% vs 59%) (Albinana-Gimenez et al., 2009; Farkas et al., 2018a; Haramoto et al., 2010; Jurzik et al., 2010; Rusiñol et al., 2015). However, detection rates and concentrations of AdV and JCV were similar in highly polluted rivers close to the wastewater discharge points near Barcelona, Spain (100%, 10^3-10^4 gc/l) and Rio de Janeiro, Brazil (100%, 10^2-10^5 gc/l) (Calgua et al., 2013). Taken together, our analysis shows that these indicator viruses are present in wastewater-polluted surface freshwater at high concentrations, which enables the accurate detection of the viruses and the comparative analysis of the rate of pollution (Crank et al., 2019; Zhang et al., 2019).

Culturable bacteriophages were also present in surface freshwater (Figure 2; Table 2). FRNAP-II were detected most frequently with concentrations up to 10^6 pfu/l followed by FRNAP-III and phages infecting *Bacteroides* (up to 10^5 pfu/l). FRNAP-II concentrations correlated with AdV concentrations in river water in France (Ogorzaly et al., 2009) and with AdV, norovirus, astrovirus and rotavirus in tropical freshwater samples in Singapore (Vergara et al., 2015). To date, no comparative studies have been done to compare enteric viruses and phages infecting *Bacteroides* spp. in surface freshwater. More research is essential on the prevalence of culturable human gut associated phages to assess their usefulness as indicators.

3.5.2 Indicators in seawater

As for freshwater environments, similar viral indicator trends have been observed in coastal waters where wastewater contamination is present. PMMoV was present at higher concentrations (10^2-10^5 gc/l) than PyV (10^2 gc/l) in coastal water at Miami, Florida (Symonds et al., 2016) and crAssphage was also present at higher concentrations (10^3-10^5 gc/l) than AdV (10^2-10^4 gc/l) and JCV (10^2-10^3 gc/l) in seawater collected at Conwy, Wales (Farkas et al., 2018a). AdV also had higher concentrations than PyV in seawater collected at Rio de Janeiro and Santa Caterina, Brazil (10^2-10^5 gc/l vs 10^1-10^3 gc/l), Florianopolis, Brazil (10^3-10^7 gc/l vs <10 gc/l), North Wales (10^2-10^4 gc/l vs 10^2-10^3 gc/l), and Catalonia, Spain (10^1-10^5 gc/l vs 10^0-10^2 gc/l) (Dias et al., 2018; Farkas et al., 2018b; Moresco et al., 2012; Rusiñol et al., 2015). CrAssphage, PMMoV and AdV and PyV are usually present up to 4 $\log_{10}$ higher concentrations in seawater than hepatitis A virus, norovirus and sapovirus (Dias et al., 2018; Farkas et al., 2018b; Fongaro et al., 2015; Moresco et al., 2012; Rusiñol et al., 2015; Symonds et al., 2018), however, one study found that the concentration of indicators and norovirus GII, rotavirus and sapovirus were similar, approx. 10^4 gc/l, in seawater collected at the Tahiti coast (Kaas et al., 2018). BacGB124P, BacRYC2056P and BacGA17P were also found in seawater at concentrations up to 10^4 pfu/l (Olalemi et al., 2016), however, these concentrations were not compared with enteric viruses. To date, FRNAP-II/III concentrations have not been measured in seawater samples. Based on the data reviewed here, PMMoV, crAssphage and AdV are suitable markers for wastewater contamination in seawater.

3.5.3 Indicators in groundwater

Very few studies have evaluated the concentration of enteric viruses and viral indicators in groundwater. AdV, JCV, AiV, PMMoV, BacGB124P and BacARABA84P were detected in polluted groundwater in the USA (Arizona and Colorado) and Vietnam at very low concentrations (Albinana-Gimenez et al., 2009; Betancourt et al., 2014; Kuroda et al., 2015), hence the concentrations cannot be compared (Figure2; Table 2). Future studies may include the efficient concentration of high volumes (> 100 l) of groundwater to accurately determine viral concentrations and the associated risks.

3.5.4 Persistence of indicators in water

Understanding how long pathogenic and indicator viruses survive in the environment is crucial for accurate risk assessment and management. The mechanisms and factors influencing viral decay, such as virus type, temperature, microbial activity, pH, water type/conductivity, UV/sunlight radiation and the presence of solid/organic matter, have been assessed (Jin and Flury, 2002; Rzeżutka and Cook, 2004; Verbyla and Mihelcic, 2015). Many studies have shown that enteric viruses are more stable in the aquatic environment than traditional indicators, such as coliform bacteria and coliphages (El-Senousy et al., 2014; Fattal et al., 1983; Keswick et al., 1982; Muscillo et al., 2008; Ogorzaly et al., 2010; Wait and Sobsey, 2001).

FRNAP are easily cultured, and their persistence in surface waters has been studied in both surface freshwaters and seawater (Hata et al., 2016; Muniesa et al., 2009; Ravva and Sarreal, 2016; Yang and Griffiths, 2013). In general, FRNAP-I has been found to be the most persistent followed by FRNAP-II, FRNAP-III and then FRNAP-IV. Using simulated sunlight, Flannery et al. (2013 b) studied the effect of solar radiation on the persistence of FRNAP-II and norovirus in seawater. The reductions in RT-qPCR detectable viruses was similar for norovirus and FRNAP-II under both summer and winter sunlight conditions. However, it took between 81% and 88% longer for a 90% reduction in RT-qPCR detectable FRNAP-II than for infectious FRNAP-II. This highlights again the need to consider infectivity when studying viral persistence in the environment. Brion et al. (2002) also studied the survival of different FRNAP genogroups in surface water. Environmental isolates of FRNAP-II had the highest variability in survival between isolates, while FRNAP-III had the lowest variability in survival between isolates. They concluded that FRNAP-III is suited to

determining whether there had been recent contamination to a water body by human faeces. In contrast, FRNAP-II was more suited to indicating contamination by distant or sporadic human source contamination.

Due to its easy molecular detection and relatively straightforward *in vitro* culturing, the survival of AdV has also been well-studied. AdVs have shown 1-2 $\log_{10}$ reduction in infectivity in raw and sterilised groundwater and surface water over 120-180 days (Ogorzaly et al., 2010; Rigotto et al., 2011). In seawater, the decrease of viral infectivity was more rapid than in groundwater, with 1.2-1.4 $\log_{10}$ AdV reductions in 28 days (Enriquez et al., 1995) and sunlight significantly enhancing degradation at a rate of at least 2 $\log_{10}$ reduction per day (Liang et al., 2017). AdVs were more stable in groundwater and surface water than poliovirus, rotavirus and hepatitis A virus (El-Senousy et al., 2014; Enriquez et al., 1995). Ogorzaly et al. (2010) showed that AdV persists for longer in ground water than both MS2 (FRNAP-I) and GA phages (FRNAP-II). This difference in survival and persistence was greatly increased with an increase in temperature from 4°C to 20°C. These studies highlight the stability of AdV compared to other viruses, however, these studies were conducted in laboratory experiments and the viral stability may differ in field conditions.

PyV has also been shown to be as resistant to sunlight in seawater as AdV (Ahmed et al., 2019b; Liang et al., 2017), however, the monitoring experiments detailed in Section 3.5.2 suggest that PyV degrades in water more rapidly than AdV. In contrast, crAssphage proved to be as persistent as AdV and PyV in coastal bathing water (Ahmed et al., 2019b). The temporal decay of AiV, PMMoV and culturable bacteriophages infecting *Bacteroides* is not yet known and should be investigated and compared with AdV decay to determine their usefulness as indicators. The comparison of the mechanisms of decay of PMMoV and the other viral indicators would be especially interesting due to the differences in the structure of the virions (tubular vs. icosahedral).

3.6 Criterion 6: Global distribution and temporal stability

All potential indicator viruses reviewed here have been detected in environmental waters, wastewater or stool samples of individuals in Asia, Europe, Australia, Africa and the Americas, highlighting the global distribution of these viruses (Cinek et al., 2018; Fratini et al., 2014; Friedman et al., 2009; Guido et al., 2016; Jofre et al., 2014; Kitajima and Gerba, 2015; Rames et al., 2016; Schaper et al., 2002; Symonds et al., 2018).

In the reviewed studies, the indicator viruses were detected and quantified in 31 countries (including 10 US states), with the majority of studies conducted in the US, Brazil, Western Europe, Japan and Australia (Figure 1; Table 2). While the available data suggest that these viruses are distributed globally, very limited information on enteric and indicator virus quantities is available from developing countries (e.g. India, Northern Asia and most African countries) (Table 2). To date, none of these viruses have been studied in water from Antarctica, where they could point towards contamination of pristine areas by research scientists, or long-distance dispersal.

During long-term monitoring surveys, in the Katsura River to the west of Kyoto, Japan, FRNAP-II was shown to have very limited seasonality, with similar levels in the winter and summer months (Hata et al., 2016). However, in a tributary of the Uji River to the South of Kyoto, FRNAP-II was detected only during winter. FRNAP-III was also found to be more prevalent during winter at both sites, a trend also observed in effluent from Johkasou effluent by Setiyawan et al. (2013). Culturable phages infecting *Bacteroides* showed no seasonal changes in their concentration in river water in the UK (Ebdon et al., 2007) and in wastewater collected in seven US states (McMinn et al., 2014) and in Brazil (Prado et al., 2018).

In the studies reviewed, crAssphage, AdV and PyV showed no seasonal changes in concentrations in untreated and treated wastewater, river and seawater samples (Carducci and Verani, 2013; Farkas et al., 2018a, 2018b; Fumian et al., 2013; Iaconelli et al., 2017; Masclaux et al., 2013; Qiu et al., 2015; Rusiñol et al., 2015; Schmitz et al., 2016). AiV and PMMoV also showed stable titres in treated and untreated wastewater over a year (Iaconelli et al., 2016; Kitajima et al., 2014; Myrmel et al., 2015; Schmitz et al., 2016), however, peak concentrations for AiV were noted in wastewater in Japan during winter and spring (Kitajima et al., 2013). PMMoV showed no seasonality in river water either (Haramoto et al., 2013; Rosario et al., 2009). Higher AdV concentrations were observed in treated wastewater collected in Wales during summer than in winter and spring, which was most likely due to dry weather and a transient increase in population due to tourism in the summer months (Farkas et al., 2018b). Furthermore, higher AdV concentrations were detected in untreated wastewater in Norway during January-March compared to the concentrations observed during April-December (Myrmel et al., 2015). The prevalence of AdV was also

higher during autumn-winter than during the spring-summer in wastewater collected in Egypt (Elmahdy et al., 2019). Similarly, AdVs were detected at low concentrations during the summer and autumn months in river water samples collected in Japan and in Germany, respectively (Hamza et al., 2009b; Kishida et al., 2012), probably due to dry weather conditions. Overall, these findings suggest that the indicators are detectable and quantifiable throughout the year, which enables the continuous evaluation of wastewater contamination. The current data imply that precipitation has more effect on viral loads than temporal changes in the number of infections. Nonetheless, this should be further investigated by comparing epidemiological data, viral loads in wastewater and precipitation over several years.

In terms of fine-scale temporal variability, the effect of rainfall on virus concentrations in surface water is variable. On one hand, a decrease in virus concentrations in surface water has been reported due to dilution of the water body (Grøndahl-Rosado et al., 2014b). In contrast, a number of studies have shown association between precipitation and elevated enteric viral concentrations in water (Ebdon et al., 2007; Wicki et al., 2015). In regions with combined sewers, much of this increase in contamination is likely due to the additional wastewater input via CSOs and storm water drainage.

CSOs discharge largely untreated (screened, partially settled or untreated) wastewater into the environment. This almost certainly results in higher numbers of enteric viruses being discharged into receiving waters than would otherwise be the case from fully treated sewage effluents (Fong et al., 2010; Hata et al., 2014). Furthermore, relatively few CSOs have spill duration monitoring and almost none have microbiological or chemical monitoring requirements. Therefore, in most cases the input of contamination can only be monitored via the surveillance of wastewater-derived contaminants in water bodies.

4. Conclusions and future research

The viruses reviewed here have all been shown to have potential to indicate wastewater-derived pollution in the aquatic environment (Table 3). Due to their wide distribution, they may be implemented in water quality risk assessments worldwide. The major advantage of enteric viral indicators (AdV, PyV, AiV) is that they are human specific, hence their use as indicators enables us to track human-derived contamination

exclusively. In addition, crAssphages and other phages, which infect commensal bacteria associated with human gut, and PMMoV, which is a plant virus accumulating in the human gut due to the consumption of infected plant-derived food, are also associated primarily with domestic wastewater contamination. A major advantage of phages and PMMoV is that they are not infectious to humans and hence their detection and culturing in the laboratory pose no risk of infection to the operators. FRNAP-II and FRNAP-III have also been shown to be useful in determining human sources of viral contamination due to their prevalence in human waste. However, due to the non-specific nature of their natural *E. coli* hosts, it is not certain what the reason is for the specific prevalence in human waste relative to FRNAP-I and FRNAP-IV. As such, it is unclear how well these can be applied globally and indeed how stable that relationship is.

The viruses reviewed here can be easily detected by qPCR-based methods, however, no such assay has been developed yet for culturable phages infecting *Bacteroides* spp. When using molecular methods, DNA viruses (AdV, PyV and crAssphage) may be easier and more affordable to monitor than RNA viruses (AiV, PMMoV, FRNAP). The infectivity of FRNAP can be easily studied using a simple and rapid plaque assay. Furthermore, the infectivity state of AdV can also be monitored using ICC-qPCR. Infectivity assays are also available for PyV, AiV and crAssphage, however, the usefulness of those assays in environmental setting have not been critically evaluated. Furthermore, there are emerging technologies, such as isothermal amplification, biosensors and microfluidics approaches, which may be useful for the routine monitoring of viruses in the environment (Farkas et al., 2020). In some cases, these may offer the potential for near real-time reporting of viral concentrations in water, however, their applicability still needs to be critically evaluated from a scientific, practical and economic perspective. This is particularly the case for *in situ* devices where biofouling, cross-reactivity and sensor drift represent major problems when translating technologies developed in the laboratory to the field (Lin and Li, 2020).

Here, the review of global studies suggests that AdVs, AiV, FRNAP-II, FRNAP-III, crAssphage and PMMoV are detected more frequently and at high concentrations in wastewater and within polluted water bodies than the other indicators reviewed. PyVs are also present in wastewater at high concentrations, however, they are less prevalent in the environment than AdV (Albinana-Gimenez et al., 2009; Bortagaray et al., 2019;

Dias et al., 2018; Haramoto et al., 2010; Moresco et al., 2012), suggesting rapid degradation. Similarly, while FRNAP-II and FRNAP-III are initially found at high concentrations in wastewater, it is likely that they degrade more rapidly in the environment than AdV. The concentration of BacGB124P, BacGA17P and BacARABA84P was also high in wastewater and have been found in wastewater polluted environments, however, only a limited number of studies have been conducted to date on viral decay prompting the need for more research in this area.

Based on their ease of detection, high concentrations in wastewater and environmental persistence, our review suggests that AdVs are the most useful viral indicators of wastewater contamination. However, AiV, crAssphage and PMMoV also show potential. More research is essential to evaluate the usefulness of these viruses and indicators. Future research should therefore focus on:

(i) Careful monitoring of the association of crAssphage and PMMoV with non-human contamination.

(ii) Monitoring the concentration and persistence of AiV, crAssphage and PMMoV in the aquatic environment, especially in groundwater and in seawater. The effect of extreme weather events on viral concentrations should also be investigated.

(iii) Development of a simple and rapid standard operating procedure for concentrating and detecting viruses from water to facilitate the accurate detection of selected indicator virus(es).

(iv) The development of multiplex qPCR assays to simultaneously detect a panel of the best markers, potentially tailored to differences in geographical diversity (particularly for PMMoV).

(v) Critical evaluation and application of new and emerging rapid approaches for viral surveillance.

(vi) Survival and maintenance of infectivity monitoring of AiV, crAssphage and PMMoV in wastewater and in the water environment. For that, the usefulness of infectivity assays for these viruses should be developed and evaluated.

(vii) Undertake comprehensive field campaigns in areas where data is not available (e.g. Africa, Asia, Oceania) to validate the use of viral indicators as an effective way to monitor wastewater pollution.

(viii) Use these viral indicators to validate current mathematical models which predict viral dispersal and which are used for risk assessment purposes.

(ix) Better establish the relationship between viral indicators and wastewater pollution to enable the development of legislative standards for viral contamination of waterbodies.

A greater understanding of the fate and behaviour of these viruses will allow them to be routinely implemented for water quality monitoring and for viral risk assessment. With a standardised protocol for the detection and quantification of proposed indicators, viral contamination can be efficiently addressed by regulators and hence the number of waterborne and foodborne viral diseases can be reduced, ultimately enhancing global human health.

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Contributors

KF and DIW conducted the literature search and the collection of data on viral concentrations. All authors contributed to structuring and writing the article.

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Table 1. Human pathogenic viruses detected in the aquatic environment

Family	Genus	Virus types found in water	Structure			Symptoms	Zoonotic	Reference
			Capsid	Genome	Size			
Adenoviridae	*Mastadenovirus*	Mastadenovirus A-F	Icosahedral	dsDNA	70-90 nm	Gastroenteritis*, respiratory illness, ear infection, conjunctivitis	No	King et al., 2009
Anelloviridae	*Alphatorquevirus*	Torque teno virus	Icosahedral	ssDNA	30 nm	Unknown, hepatitis*	Yes	King et al., 2009
Astroviridae	*Mamastrovirus*	Astrovirus	Icosahedral	ssRNA+	28-30 nm	Gastroenteritis	Potentially	De Benedictis et al., 2011; King et al., 2009
Caliciviridae	*Norovirus*	Norovirus GI, GII	Icosahedral	ssRNA+	35-40 nm	Gastroenteritis	No	King et al., 2009
	Sapovirus	Sapovirus GI, GII				Gastroenteritis	No	King et al., 2009
Circoviridae	*Circovirus*	Human-associated circovirus	Icosahedral	ssDNA	15-25 nm	Unknown*	No	Breitbart et al., 2017
Hepeviridae	*Orthohepevirus*	Hepatitis E virus type 1-4	Icosahedral	ssRNA+	27-34 nm	Acute hepatitis*	Yes	Purdy et al., 2017
Papillomaviridae	various	assorted papillomaviruses	Icosahedral	dsDNA	55 nm	Genital tract infection, cancer*	No	Van Doorslaer et al., 2018
Parvoviridae	*Bocavirus*	Human bocavirus type 1-4	Icosahedral	ssDNA	22 nm	Gastroenteritis and respiratory disease	No	King et al., 2009
Picornaviridae	*Kobuvirus*	Aichivirus A-B	Icosahedral	ssRNA+	30-32 nm	Gastroenteritis*	No	Zell et al., 2017
	Cosavirus	Cosavirus A				Gastroenteritis*	No	
	Enterovirus	Coxsackievirus B Enterovirus A-D Poliovirus type 1-3				Gastroenteritis, mild meningitis, encephalitis, myelitis, myocarditis, conjunctivitis*	No	
	Hepatovirus	Hepatitis A virus				Gastroenteritis, hepatitis	No	
Polyomaviridae	*Alpha-polyomavirus*	MC polyomavirus	Icosahedral	dsDNA	40-45 nm	Cancer*	No	Moens et al., 2017
	Beta-polyomavirus	BK polyomavirus JC polyomavirus	Icosahedral	dsDNA	40-45 nm	Respiratory, urinary tract and skin infection, cancer*	No	Moens et al., 2017
Reoviridae	*Reovirus*	Rotavirus A	Icosahedral	dsRNA	60-80 nm	Gastroenteritis	Potentially	Cook et al., 2004; King et al., 2009

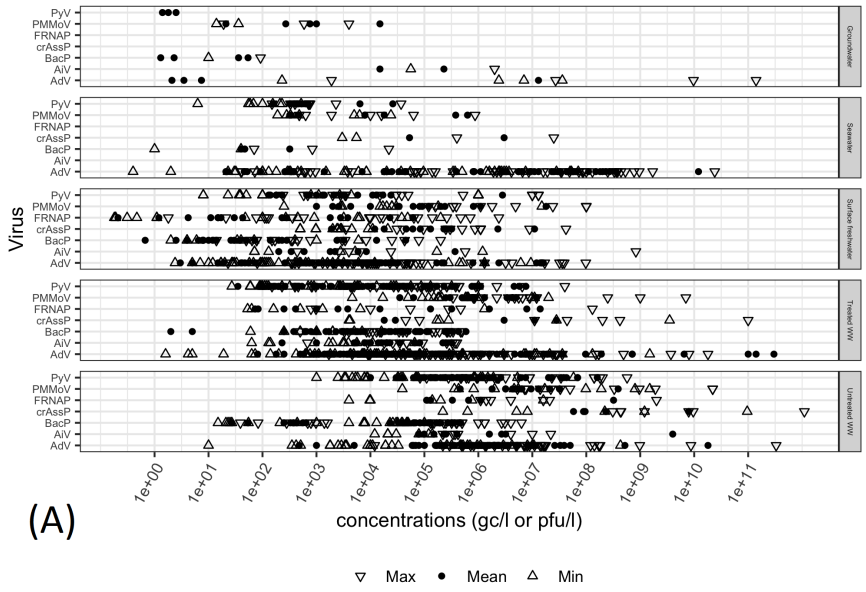
*May be asymptomatic in otherwise healthy individuals

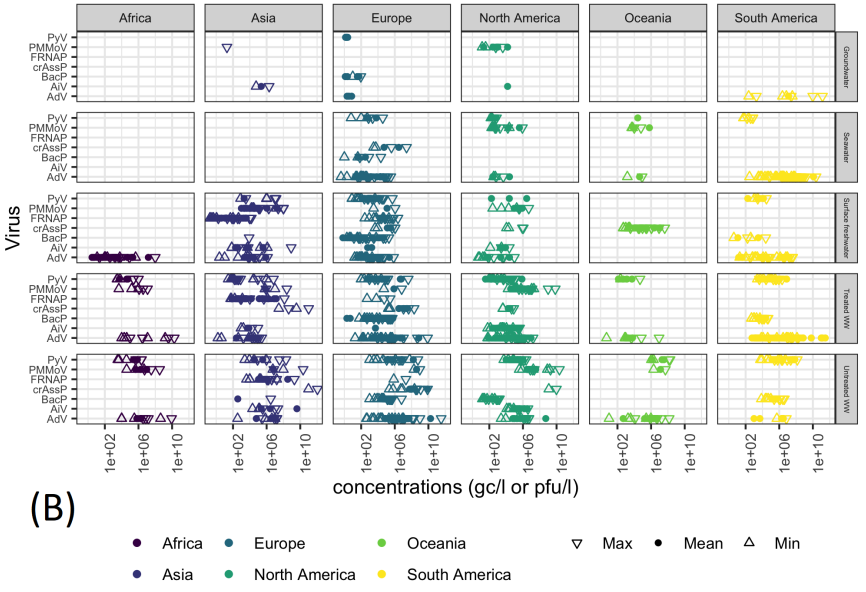
Table 2. Number of reviewed studies for each indicator at each region.

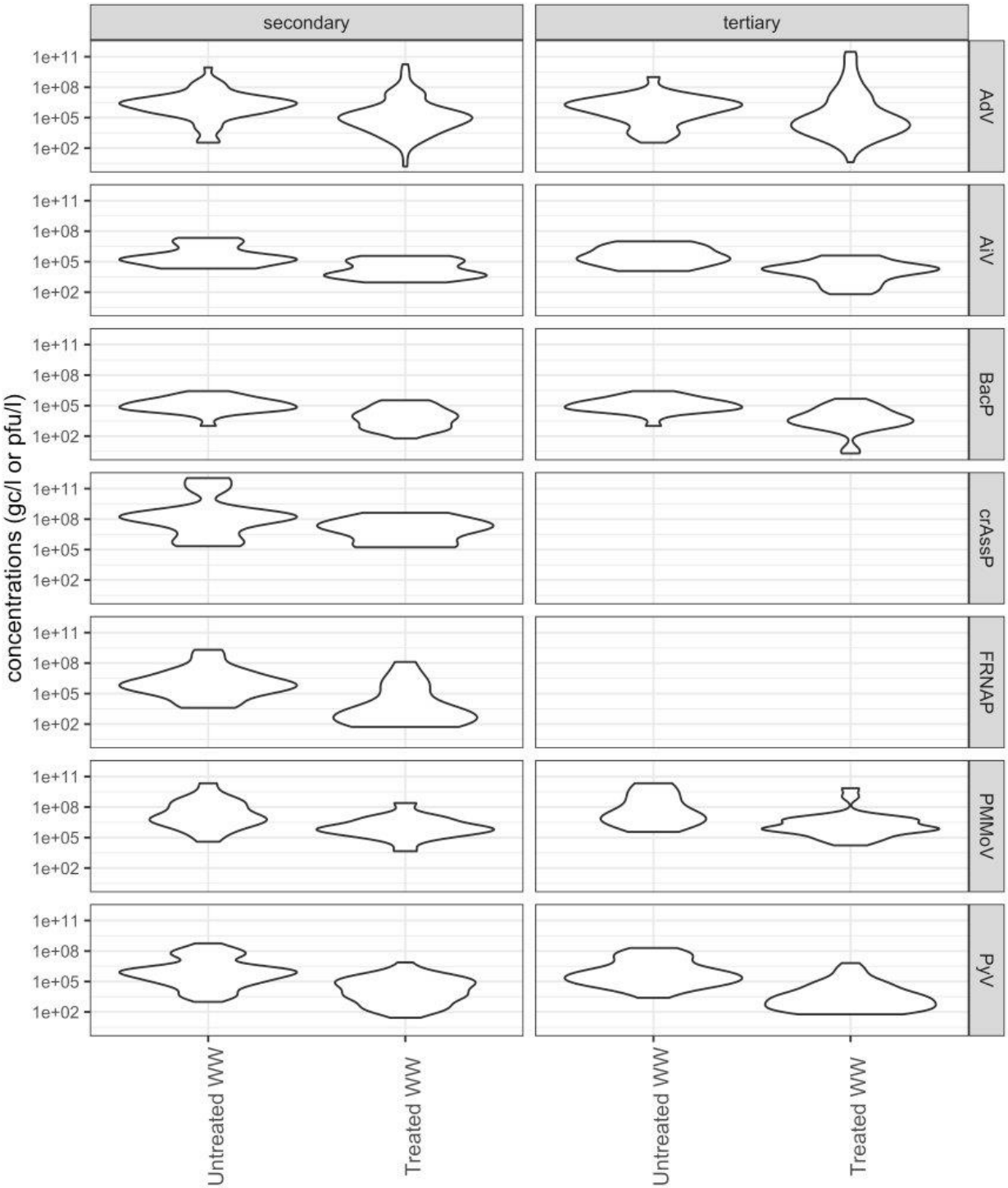
		North America	South America	Africa	Europe	Asia	Oceania	Global detection rate
AdV	Raw wastewater	7	2	2	13	3	5	94% (772/823)
	Treated wastewater	10	7	2	13	3	3	86% (1223/1436)
	Surface freshwater	4	5	4	7	4	0	65% (835/1283)
	Groundwater	1	2	0	1	1	0	65% (40/62)
	Seawater	2	6	0	4	0	0	60% (229/381)
	Total	13	16	5	20	5	4	76% (3099/3985)
PyV	Raw wastewater	5	5	1	7	2	3	93% (542/581)
	Treated wastewater	6	5	1	6	2	2	68% (608/892)
	Surface freshwater	1	2	0	6	2	0	52% (326/631)
	Groundwater	0	0	0	1	0	0	48% (10/21)
	Seawater	4	0	0	2	2	1	24% (83/350)
	Total	9	7	1	10	4	3	63% (1569/2475)
AiV	Raw wastewater	5	0	0	0	4	0	91% (92/101)
	Treated wastewater	5	0	0	1	3	0	74% (184/250)
	Surface freshwater	2	0	0	1	3	0	33% (77/236)
	Groundwater	1	0	0	0	0	0	55% (26/47)
	Seawater	0	0	0	0	0	0	NA
	Total	6	0	0	1	6	0	60% (379/634)
PMMoV	Raw wastewater	6	0	1	1	2	1	100% (110/110)
	Treated wastewater	6	0	1	1	2	0	99% (135/137)
	Surface freshwater	2	0	0	1	4	0	87% (278/319)
	Groundwater	1	0	0	0	0	0	72% (18/25)
	Seawater	1	0	0	0	0	1	55% (45/82)
	Total	7	0	1	1	5	1	87% (586/673)
Bacteroides phages	Raw wastewater	2	2	0	14	2	0	97% (531/549)
	Treated wastewater	0	1	0	8	1	0	75% (911/1216)
	Surface freshwater	2	1	0	4	1	0	66% (280/427)
	Groundwater	0	0	0	3	0	0	38% (48/127)
	Seawater	0	0	0	3	0	0	42% (43/102)
	Total	3	2	0	19	2	0	72% (1741/2421)
FRNAP (II/III)	Raw wastewater	0	0	0	1	3	0	73% (96/132)
	Treated wastewater	0	0	0	1	4	0	81% (219/270)
	Surface freshwater	0	0	0	2	5	0	59% (375/634)
	Groundwater	0	0	0	0	1	0	0% (0/10)
	Seawater	0	0	0	0	0	0	NA
	Total	0	0	0	3	8	0	66% (690/1046)

Table 3. Summary on how the reviewed viruses meet the criteria for wastewater indicator.

Criterion	AdV	PyV	AiV	PMMoV	FRNAP (II/III)	Culturable *Bacteroides* phages	CrAssphage
Methods used for detection in environmental samples	qPCR; ICC-qPCR; culturing	qPCR	qRT-PCR	qRT-PCR; plant infectivity assay	qRT-PCR; culturing	culturing	qPCR
Human association	Human-specific	Human-specific	Human-specific	Human waste and agricultural sites	Primarily human gut-associated	Primarily human gut-associated, have been found in animal faeces at low titres	Primarily human gut-associated, have been found in animal faeces at low titres
Concentration in wastewater (gc/l)	$1x10^1$ – $3x10^{11}$	$1x10^3$ – $6x10^8$	$1x10^4$ – $4x10^6$	$3x10^5$ – $2x10^{10}$	$4x10^3$- $2x10^9$	$1x10^1$ – $6x10^6$	$2x10^5$ – $1x10^{12}$
Log_{10} removal during wastewater treatment	0.2 – 5.5 (n=500)	0.3 – 4.2 (n=407)	0.8 – 2.7 (n=72)	0 – 2.7 (n=106)	0.1 – 3.1 (n=172)	0.5 – 5.6 (n=304)	1 – 1.2 (n=39)
Concentration in the aquatic environment (gc/l)	4 – $2x10^{10}$	1 – $1x10^7$	$7x10^1$ – $8x10^8$	$1x10^1$ – $8x10^8$	0.2-$2x10^6$	1 – $2x10^5$	$1x10^3$ – $3x10^7$
Global distribution and temporal stability	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations


PyV
PMMoV
FRNAP
crAssP
BacP
AiV
AdV
Groundwater
Seawater
Surface freshwater
Treated WW
Untreated WW
Virus
1e+00
1e+01
1e+02
1e+03
1e+04
1e+05
1e+06
1e+07
1e+08
1e+09
1e+10
1e+11
concentrations (gc/l or pfu/l)
(A)
Max
Mean
Min


Africa
Asia
Europe
North America
Oceania
South America
Virus
1e+02
1e+06
1e+10
concentrations (gc/l or pfu/l)
(B)
Africa
Asia
Europe
North America
Oceania
South America
Max
Mean
Min


secondary
tertiary
AdV
AiV
BacP
crAssP
FRNAP
PMMoV
PyV
concentrations (gc/l or pfu/l)
1e+11
1e+08
1e+05
1e+02
Untreated WW
Treated WW

Highlights

- Human mastadenoviruses are robust indicators for human-associated pollution in water
- *Bacteroides*-associated phages and crAssphage are promising indicators
- Multiple indicators should be used to assess wastewater treatment efficiency
- Survival and abundance of indicator viruses should be further assessed

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: